(12) UK Patent Application (19) GB (11) 2 239 246 (13) A

(43) Date of A publication 26.06.1991

(21) Application No 9027267.5

(22) Date of filing 17.12.1990

(30) Priority data
(31) 938MAS89 (32) 22.12.1989 (33) IN
9001105 27.03.1990 SE

(71) Applicant
Aktiebolaget Astra

(Incorporated in Sweden)

A-151 85 Sodertalje, Sweden

(72) Inventors
Chinnaswamy Jagannath
Meenakshi Balganesh
Bachally Ramasastry Srinivasa

(74) Agent and/or Address for Service
J A Kemp and Co
14 South Square, Gray's Inn, London, WC1R 5LX,
United Kingdom

(51) INT CL[5]
C12N 15/31, A61K 39/04, C07K 7/06 7/08 13/00
G01N 33/53 // (C12N 15/31 C12R 1:32)

(52) UK CL (Edition K)
C3H HA5 H310 H311 H316 H339
C6Y Y146
U1S S1337 S2419

(56) Documents cited
Nucleic Acids Res. 1989, 17(3), 1249
Infect. Immun. 1989, 57(8), 2481-2488
Infect. Immun. 1989, 57(10), 3123-3130
J. Gen. Microbiol. 1989, 135, 931-939
J. Bacteriol. 1987, 169(3), 1080-1088

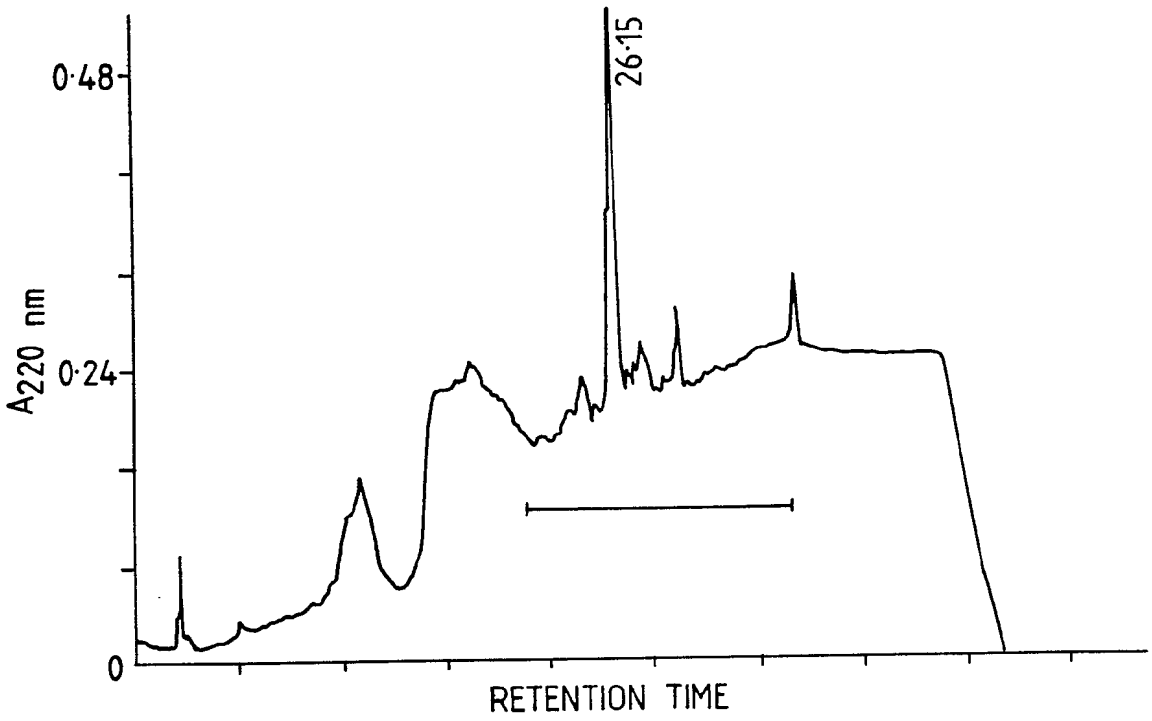
(58) Field of search
UK CL (Edition K) C3H HA3 HA5 HB7P HC2
INT CL[5] C07K, C12N
Online databases: WPI, CHABS, DIALOG/BIOTECH

(54) Protein antigen from Mycobacterium tuberculosis

(57) A 17 KDa protein has been isolated from *M. tuberculosis* (South Indian Isolate -1) and sequenced. This 131 amino acid protein and fragments thereof including amino acids 68-77, 91-101 and/or 107-122, antibodies to the protein and the fragments and DNA encoding the protein or fragments, or DNA hybridisable to such DNA are of interest in the immuno diagnosis, therapy and vaccination in relation to human tuberculosis.

GB 2 239 246 A

Fig. 1.



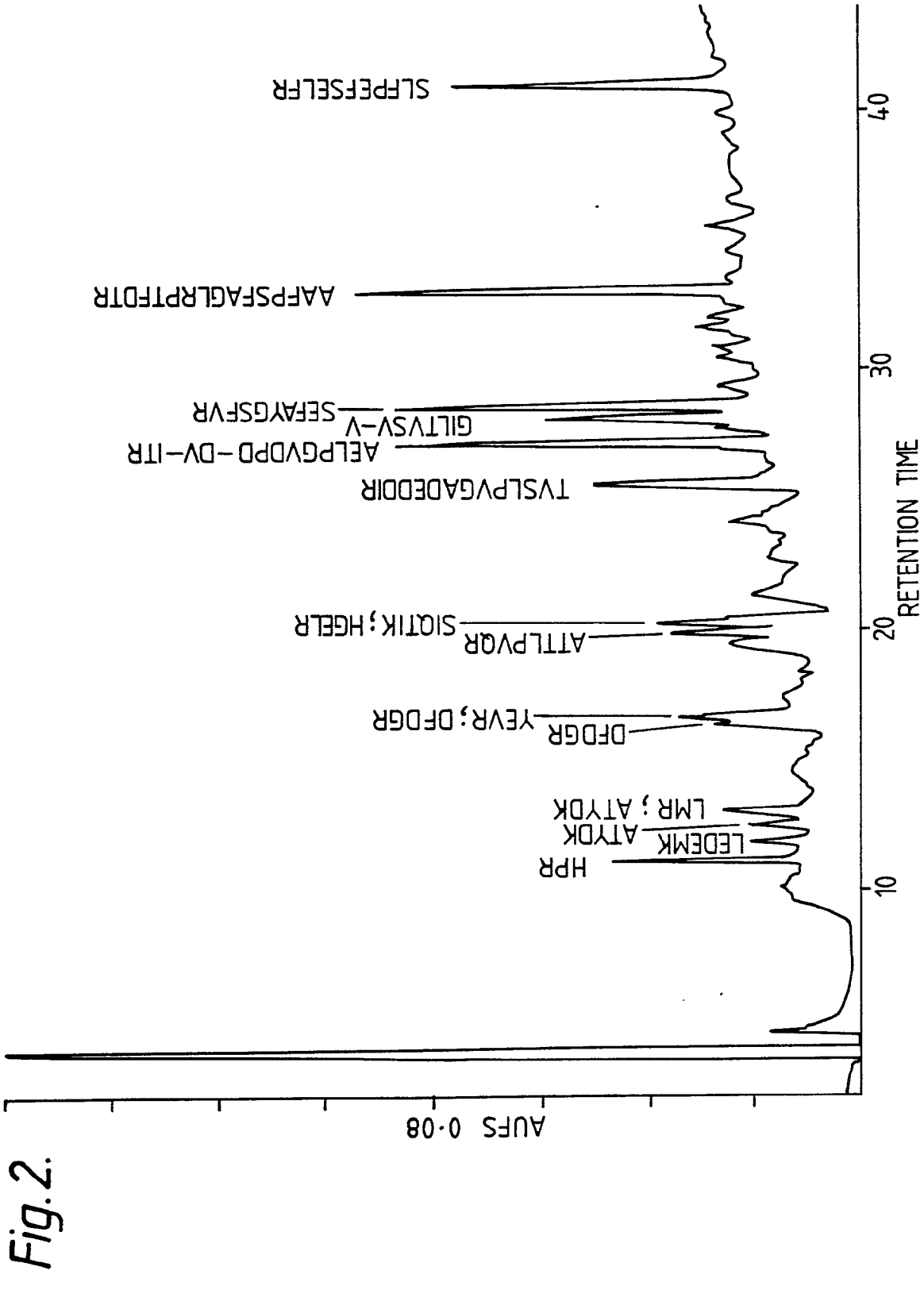

AUFS 0·08
RETENTION TIME
10
20
30
40
HPR
LEDEMK
ATYDK
LMR ; ATYDK
DFDGR
YEVR ; DFDGR
ATTLPVQR
SIQTIK ; HGELR
TVSLPVGADEDDIR
AELPGVDPD-DV-ITR
GILTVSV-V
SEFAYGSFVR
AAFPSFAGLRPTFDTR
SLFPEFSELFR

Fig. 2.

Fig. 3.

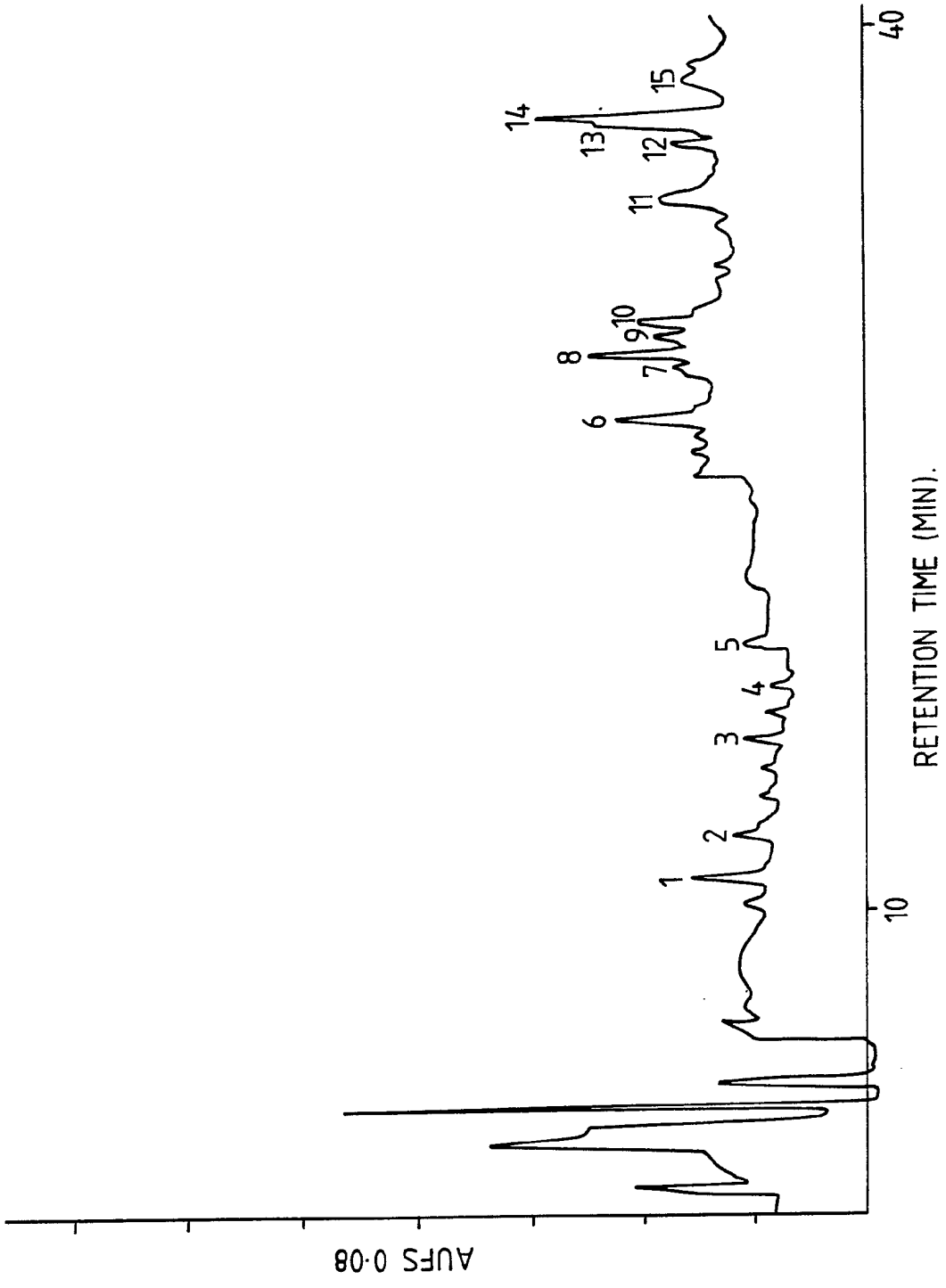
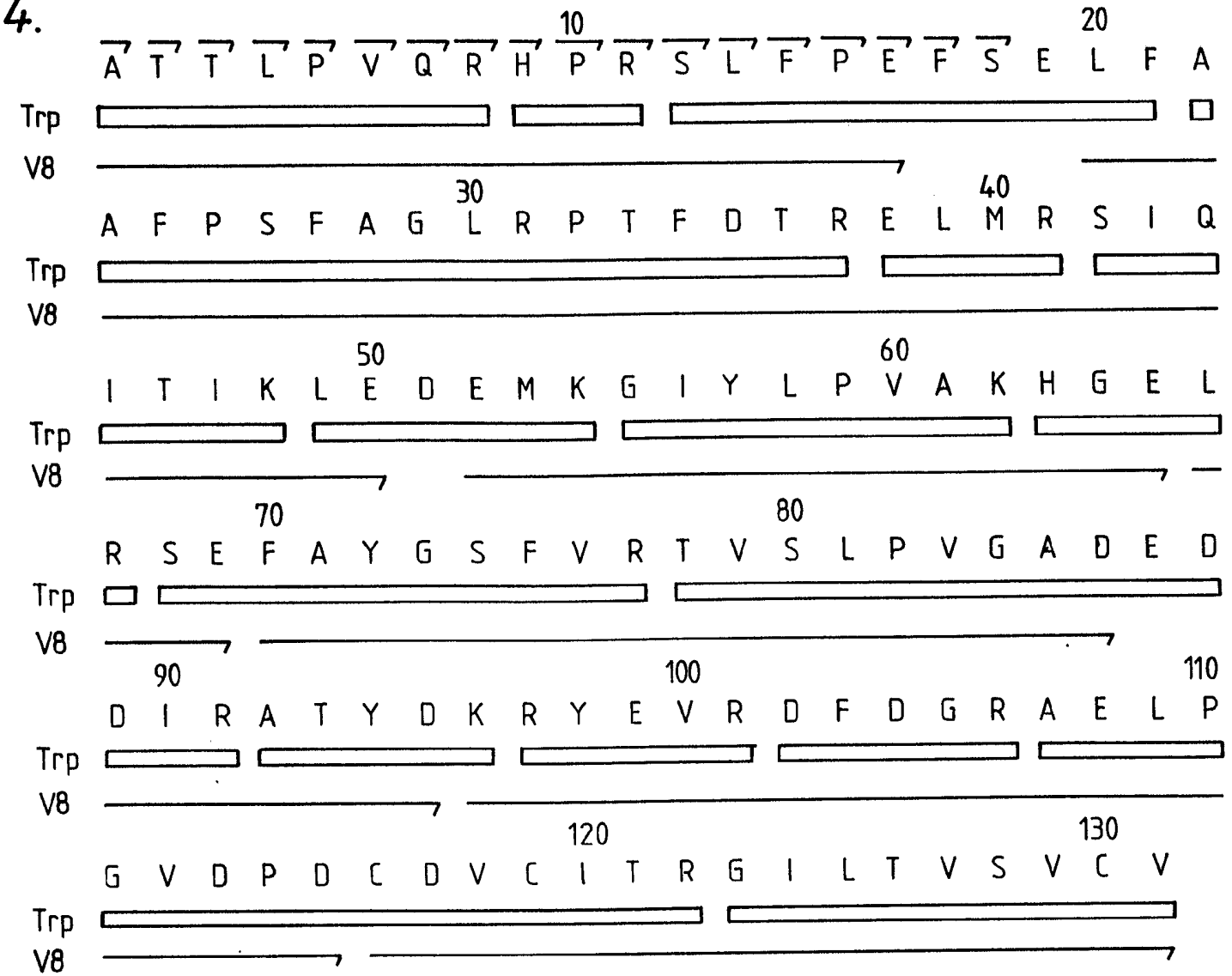
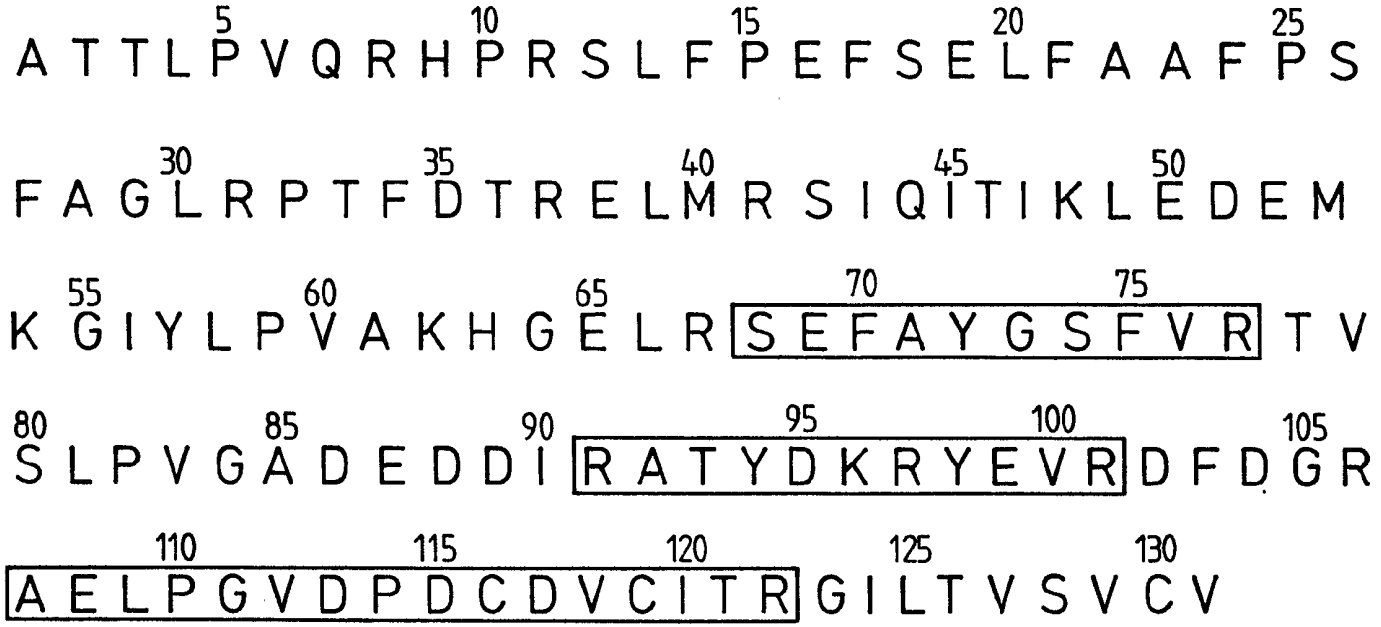


Fig.4.


10
20
A T T L P V Q R H P R S L F P E F S E L F A
Trp
V8
30
40
A F P S F A G L R P T F D T R E L M R S I Q
Trp
V8
50
60
I T I K L E D E M K G I Y L P V A K H G E L
Trp
V8
70
80
R S E F A Y G S F V R T V S L P V G A D E D
Trp
V8
90
100
110
D I R A T Y D K R Y E V R D F D G R A E L P
Trp
V8
120
130
G V D P D C D V C I T R G I L T V S V C V
Trp
V8

4/5

Fig.5.


5 10 15 20 25
A T T L P V Q R H P R S L F P E F S E L F A A F P S
30 35 40 45 50
F A G L R P T F D T R E L M R S I Q I T I K L E D E M
55 60 65 70 75
K G I Y L P V A K H G E L R S E F A Y G S F V R T V
80 85 90 95 100 105
S L P V G A D E D D I R A T Y D K R Y E V R D F D G R
110 115 120 125 130
A E L P G V D P D C D V C I T R G I L T V S V C V

2239246

"NEW METHODS FOR DIAGNOSIS OF TUBERCULOSIS"

NEW METHODS FOR DIAGNOSIS OF TUBERCULOSIS

1. Summary of the invention

Disclosed are the immunochemical properties of a 17 kDa protein antigen fractionated, purified and sequenced for amino acids from Mycobacterium tuberculosis (South Indian isolate, SII 1) which causes human tuberculosis worldwide among 16 million patients. The 17 kDa protein antigen which has an N-terminus of A T T L P V Q R (aa 1-8) has atleast three specific antibody binding epitopes located on linear peptides of sequences, R A T Y D K R Y E V R (aa 91-101) and S E F A Y G S F V R (aa 68-77) which were useful in a micro ELISA for the early diagnosis of human tuberculosis by the detection of specific antibodies. The 17 kDa protein antigen which was mitogenic for human tuberculous peripheral blood lymphocytes was found to carry three predicted T-cell epitopes on linear peptides of sequences, S E F A Y G S F V R (aa 68-77) and A E L P G V D P D C D V C I T R (aa 107-122). The 17 kDa antigen of M. tuberculosis (SII 1) which thus had both B and T cell reactive properties was found to contain 131 amino acids and potentially applicable in the immuno diagnosis, immuno therapy and immuno prophylaxis of human tuberculosis.

2. Field of invention

The present invention relates to a novel 17 kDa protein antigen of Mycobacterium tuberculosis (South Indian Isolate SII 1) and certain peptide fragments derived therefrom, and to the use of the said antigen, and of the peptide fragments derived therefrom, in immuno diagnosis, immunotherapy and immuno prophylaxis of human and experimental tuberculosis. The invention also relates to the DNA sequence coding for the said 17 kDa antigen, to DNA sequences coding for the said peptide fragments derived from the 17 kDa antigen and to the DNA and RNA probes constructed on the basis of the protein sequence of the 17 kDa antigen including the sequences of the peptide fragments of the 17 kDa antigen. A major field of use is the use of 17 kDa antigen and peptide fragments thereof in immuno diagnosis of tuberculosis. A further field of use is the use of the 17 kDa antigen or of the said peptide sub structures thereof for the preparation of a vaccine against tuberculosis. A further field of use is the use of the 17 kDa antigen or its sub structure peptides for the detection of T cell proliferation by skin tests or invitro tests in man. This last mentioned field of use is of importance in the possible treatment of human cancer by the boosting of cellular immunity. A further field of use is the use of the 17 kDa antigen or its sub structure peptides for the laboratory production of cellular growth factors and enzymes.

3. Background of invention

Human tuberculosis caused by M.tuberculosis is an important chronic debilitating disease occuring worldwide affecting about 16 million people. While it is prevalent mostly in under developed and developing countries, the recent epidemic of Acquired Immuno Deficiency Syndrome (AIDS) among developed countries has posed a problem of secondary infection with mycobacteria including M.tuberculosis.

Precise and confirmative diagnosis of human TB enables early treatment for which powerful antibiotics are available, although in many countries drug resistance has become a problem. Nevertheless, early diagnosis of TB means effective chemotherapy and thus elimination of the transmission of live bacilli from pulmonary TB patients.

Conventional diagnosis of TB depends upon the clinical and radiological findings, microscopy of tuberculous specimen for bacilli, and bacteriological isolation in culture of M.tuberculosis.

Global control of TB has not yet been established because of the inadequacy of the presently available methods of diagnosis.

Thus many clinical features of TB are not specific to TB alone and a study in India (source: National Tuberculosis Insitutute, Bangalore, India) revealed that only 30% of the X-ray suspects eventually developed TB although, in many countries including India, all X-ray suspects are put on anti tuberculous chemotherapy.

The microscopy of tuberculous specimen is not easy under field conditions and atleast 10^4 bacilli/ml are required for effective screening. Many tuberculous specimens like cerebro spinal fluids from tuberculous meningitis infrequently contain bacilli. Further, bacteriological culture generally takes 6 to 8 weeks and is expensive as a routine diagnostic measure.

The widely used tuberculin skin test lacks sensitivity and specificity and takes about 3 days for completion. Since chemotherapy of TB requires compliance for atleast 6 months, many patients who are irregular in treatment develop drug resistance and transmit live bacilli. Finally, the traditional BCG vaccination has now been found to give varying levels of protection depending upon geographic regions.

Consequent to these factors, many investigators including the WHO recommend that early diagnosis of TB should be considered as a priority area of research and development.

Mycobacteria are powerful immunogens for man and animals, as evidenced by their use in immuno adjuvants to boost immune responses. Thus, many antigens derived from M.tuberculosis have been known to induce the formation of specific antibodies and proliferative lymphocytes among TB patients (Ivanyi et al., 1988) and experimental animal models. The detection of an antibody response in TB has a potential application in early diagnosis. Likewise, the study of M.tuberculosis specific T lymphocytes has application in early diagnosis by skin tests and development of protective vaccines.

Thus the focus of research in TB pertaining to these aspects lies in the identification and synthesis of M.tuberculosis specific antigens.

4. Prior Art

Many investigators have identified protein antigens of M.tuberculosis which have potential immuno diagnostic or immuno prophylatic application. The N-terminal and internal amino acid sequences of many of these antigens have been published. The N-terminal amino acid sequences of some of these are presented in Table 1.

Table I: M.tuberculosis protein antigens identified by N-terminal amino acid sequences.

Investigators	Antigens and N-terminus
Shinnick et al, 1987	65 kDa: R G C R H P V
Yamaguchi et al, 1987	MPB 57: M A K F N I K P L
Pattorroyo et al, 1987	13 kDa: A K V N I
	18 kDa: G D L V G P G A E
	23 kDa: A P K T Y
	30 kDa: F S X P G L
	68 kDa: W M T M T
	77 kDa: G K X I A Y D G A A
Matsuo et al, 1988	30 kDa: F S R P G L P
Ashbridge et al, 1989	19 kDa: E H R V K R G L T V
Baird et al, 1989	10 kDa: A K V N I P K P
Garcia et al, 1989	70 kDa: F Q R I T R Q D L L
Borremans et al, 1989	32 kDa: F S R P G L P

To our knowledge, none of these antigens have been introduced as immuno diagnostic test products. Two of these antigens (10 kDa of Baird et al, 1989 and a homologue of 65 kDa antigen of Shinnick et al, 1987) have been tested for vaccine potency among experimental animal models where they showed poor protection against M.tuberculosis (D.W. Smith, University of Wisconsin, USA, personal communication).

An ELISA kit for diagnosing TB using A60 antigen present in all mycobacteria has been introduced by ANDA diagnostics (France). The test is thus not specific for human TB alone.

An alternative method of diagnosis of TB has been the use of DNA probes. The commercially available GEN PROBE kit (1988) is used to confirm the identity of members of M.tuberculosis complex isolated in culture and has not been used directly on clinical specimen. A positive result with this test does not rule out other mycobacteria. The DNA probe devised by Enzo Biochem (J.Clin Microbiol.1988, Dec) used specific DNA sequences of 1000 bases or more in length and claimed to be more specific. A major handicap of all DNA based probes is that whether a true positive reaction can be obtained with specimens of patients so as to avoid the laborious culture of bacilli.

Phenotypic variation in virulence is known among the M.tuberculosis strains isolated in South India (Naganathan et al 1987), the molecular basis for which is not yet established. Abou Zeid et al, (1988) found that a 13 kDa

protein antigen was present in phage type II virulent M.tuberculosis and absent in phage type I South Indian low virulent M.tuberculosis. However, the relationship between this antigen and virulence is yet to be investigated in detail.

5. The Invention :

A 17 kDa protein antigen was found to be present among the isolates of M.tuberculosis. The immuno chemical features of this antigen are disclosed under this invention.

Thus the present invention relates to:

1. The 17 kDa protein antigen of M.tuberculosis (SII 1) as defined below and certain sub structures (peptides) of 17 kDa protein antigen as defined below.

2. A DNA sequence coding for the 17 kDa antigen from M.tuberculosis (SII 1).

3. A DNA sequence coding for the sub structures (peptides) of the 17 kDa antigen from M.tuberculosis (SII 1).

4. The use of the said 17 kDa protein or the said sub structures (peptides) for the preparation of monoclonal or polyclonal antisera which react with the said 17 kDa protein antigen or sub structures thereof. The said antibodies can be raised in mammals like mice, rabbits and goats for polyclonals and in mice for monoclonals.

5. The use of the said 17 kDa protein antigen of the said sub structures (peptides) for the detection of antibodies among human and animal specimen for immuno-diagnosis. Detection methods are known in the art one employs such as ELISA, radio immuno assay (RIA) and reverse passive hemagglutination (RPHA).

6. The use of the 17 kDa protein antigen or sub structures (peptides) thereof for the treatment of tuberculosis.

7. The use of the 17 kDa protein antigen or substructures peptides thereof for the preparation of a vaccine against tuberculosis.

8. The use of the 17 kDa antigen or sub structures (peptides) thereof for the preparation of a reagent for the skin test in immuno diagnosis for tuberculosis.

9. DNA or RNA probes constructed on the basis of the protein sequence of the 17 kDa antigen or sub structures (peptides) thereof for diagnosis of tuberculosis. Such probes can be constructed by methods known in the art. Labelling of such probes can be done by known methods such as radioisotope incorporation or by non radio-active labelling use for example, biotin.

10. A method of diagnosis of human tuberculosis by interacting body fluids such as serum, CSF, pleural fluids from a patient to be diagnosed with a monoclonal antibody to the 17 kDa antigen or its sub structures (peptides) as defined in paragraph 3 above.

11. A method for diagnosis of human tuberculosis by interacting a body fluid such as serum from a patient to be diagnosed with a 17 kDa protein as defined in paragraph 5 above.

12. A method of diagnosing human tuberculosis by interacting body fluids such as sputum, serum, CSF and pleural fluids from a patient to be diagnosed with a DNA or RNA probe as defined in paragraph 9 above.

13. A method for in vitro detection of human tuberculosis which comprises contacting a sample of a body fluid such as

sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody as defined in paragraph 10 in labelled form.

14. A method for in vitro detection of human tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody as defined in paragraph 10 in labelled form.

15. A method for in vitro detection of human tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, plental fluid or serum from a patient with a monoclonal antibody as defined in paragraph 10 in labelled form.

16. A kit for performing immuno diagnosis of tuberculosis utilizing a monoclonal antibody to 17 kDa antigen as disclosed in paragraph 3 above.

17. A kit for immuno diagnosis of tuberculosis utilizing 17 kDa antigen or peptide sub structures as disclosed in paragraph 5 above.

18. A kit for diagnosing tuberculosis utilizing a DNA or RNA probes as disclosed in paragraph 9 above.

19. A micro organism expressing a 17 kDa protein or sub structures thereof as disclosed in paragraph 1 above.

20. A vaccine against tuberculosis developed on the basis of the 17 kDa antigen or sub structure peptides thereof as disclosed in paragraph 1 above. Such a vaccine can be a product of genetically engineered organisms such as Salmonella, Vaccinia virus etc.

The present invention is exemplified by but not limited to the diagnosis, therapy or prophylaxis of diseases, especially diagnosis of M.tuberculosis infection. Epidemiological screening, forensic investigations, determination of food contaminations, public health surveys, preventive medicine, veterinary and agricultural applications with regard to the diagnosis of infectious agents may be covered by this disclosure.

5.1 Fractionation and purification of 17 kDa antigen.

5.1.1 Crude sonicate antigen.

M.tuberculosis (SII 1) was cultured at 37°C for 2 weeks in Kirchner's medium and harvested bacilli were killed in cold acetone for 18 h at 4°C. The bacilli were washed thrice with saline and a suspension of 10 mg bacilli in 5 ml saline was sonicated at 40 watts output using a miniprobe of a Branson sonifier. The sonicate was centrifuged at 20,000 x g for 30 min and the supernatant was estimated for protein content (Lowry's method) prior to freeze storage at -70°C.

5.1.2 Fractionation and purification.

500 ug of crude sonicate was fractionated on 12.5% sodium dodecyl sulfate polyacrylamide gel as described by Hunkapiller and Lujan (1986). The protein bands were visualized by brief staining with coomassie brilliant blue and the 17 kDa antigen was electroeluted against 0.05 M ammonium bicarbonate with 0.1% SDS followed by electro-dialysis against 0.01 M ammonium bicarbonate with 0.02 % SDS. The eluted protein was then extracted with chloroform-methanol to remove SDS and the precipitate was dried.

The purity of the eluted protein was analysed by subjecting 5 ug of this precipitate to HPLC using Lichrosorb RP 18 column (LKB). A single peak eluted at 45% B and 26 min was found to contain immuno reactive antigen. Fig. 1 shows the HPLC profile of 17 kDa antigen.

5.2 Amino acid sequence analysis of 17 kDa antigen

5.2.1 Peptide mapping of 17 kDa antigen

Tryptic map: 30 ug of 17 kDa protein was digested with TPCK treated trypsin with an enzyme to substrate ratio of 1:50 in 0.1 M ammonium bicarbonate buffer, pH 7.8 at 37 ^{o}C for 5 h. The tryptic digest was fractionated by HPLC on RP 18 column (0.46 x 25 cm) equilibrated with solvent A (0.1% TFA in water) and the peptides were eluted with a gradient of solvent B (70% acetonitrile containing 0.085% TFA) from 0 to

65% in 60 min. The tryptic map is shown in Fig. 2.

V8 protease map: 30 ug of 17 kDa antigen was treated with staphylococcal V8 protease for 48 h in 0.07% ammonia at 37°C. The molar ratio of the enzyme to substrate was 1:25. The various peptides in the enzyme digest were purified on an HPLC column uder the conditions used for tryptic mapping. The peptide profile is shown in Fig. 3.

5.2.2 Sequence analysis of 17 kDa antigen

The amino acid sequence analysis of the protein and the peptide was done using protein sequencer model 477A (Applied Biosystems Inc., USA) with an on line PTH amino acid analyser. The sample was solubilized in 10% formic acid and fixed onto a polybrene coated (1 mg) TFA treated glass fibre disc and used for sequencing.

The first 18 amino acids from the N-terminal was determined using the whole protein. Based on the amino acid sequences of the tryptic peptides, V8 protease was selected to generate the peptides that could give the overlaps for the tryptic peptides. The alignment of both tryptic and V8 protease peptides gave the complete sequence for the 17 kDa antigen. The details of the overlaps are given in Fig. 4.

5.2.3 Amino acid composition of 17 kDa antigen

The protein has A9, C3, D11, E10, F9, G8, H2, 17, K4, L11,

M2, P9, Q2, R12, S8, T9, V11 and Y4. It is significant in not having tryptophan and asparagine. The protein is acidic in nature since it has 5 acidic amino acids (D+E=21) in excess of the total number of basic amino acids (R+K=16). The protein has 131 amino acids that account for a molecular weight of 14,762.

5.3 Immuno reactivity of 17 kDa antigen

5.3.1 Demonstration that 17 kDa antigen is immunodominant in M.tuberculosis strains.

M.tuberculosis (SII 1), M.tuberculosis ATCC 27294, M.phlei, M. smegmatis, M.kanasasii, M.avium intracellulare and M.scrofulaceum were cultured in Kirchner's medium for 2 weeks and harvested bacilli were killed in cold acetone. Sonicate antigens were prepared from each of these species as described in section 5.1.1. SDS PAGE analysis of these antigens was then carried out in 12.5% gel. Coomassie blue stained gels showed that the 17 kDa antigen was present only in M.tuberculosis strains. A rabbit antiserum raised to M.tuberculosis SII 1 was also used to probe these sonicates in Western blotting. The dominant 17 kDa band was found in the Western blot.

5.3.2 Demonstration that 17 kDa elicits antibodies among experimental animals.

Electro eluted 17 kDa antigen (10 ug in 100 ul saline) was emulsified with an equal volume of Freund's incomplete adjuvant (FICA) and used for intra peritoneal immunization of 10 BALB/c mice. Serum collected from these mice 30 days after immunization recognized a 17 kDa band in the sonicate antigen of M.tuberculosis SII 1.

Thus this experiment confirms that a polyclonal or monoclonal antibody can be produced in the mouse which recognize the protein structure of 17 kDa antigen or sub structures (peptides) thereof. Such antibodies, in particular the monoclonal antibodies can be used in an antigen detection method like the sandwich ELISA for the detection of the 17 kDa antigen or sub structures thereof among human tuberculosis specimen leading to immuno diagnosis of tuberculosis.

5.3.3 Demonstration that 17 kDa antigen reacts with human TB patient sera.

Sera derived from 24 healthy persons and 20 culture proven TB patients were titrated against the 17 kDa antigen as follows. PVC Dynatech plates were coated with 1 ug/ml PBS of electro eluted 17 kDa antigen for 24 h at 22 ^{o}C. PBS-BSA blocked plates were then titrated against duplicate (1/200) dilutions of sera which were incubated at 22 ^{o}C for 2.5 h. Washed plates received anti human IgG HRP conjugate for 1.5 h. Washed plates were then assayed with O-phenylene diamine

substrate and read at 492 nm. Table II shows that the 17 kDa antigen had a sensitivity of 70% and specificity of 85%

Table II. Micro ELISA with 17 kDa antigen on sera from TB patients and controls

Serum groups	n	ELISA +	ELISA-	Sensitivity	Specifici
Healthy people	24	4	20	--	85%
TB patients	20	14	6	70%	--

Sensitivity: Known positivity among TB patients
Specificity: Known negativity among healthy controls
ELISA + : OD 492 nm >=0.3 at 1/200 dilution (=mean + 2SD of OD 492 nm for healthy controls, n=24].

Thus this experiment confirms that the 17 kDa antigen from M.tuberculosis strains can be used in a micro ELISA system for the immuno diagnosis of tuberculosis in man.

5.3.4 Demonstration that the 17·kDa protein antigen has defined antibody epitopes

The peptide fragments derived from the 17 kDa antigen by tryptic digestion (section 5.2.1, Fig.2) were individually titrated against sera from healthy persons and TB patients as in section 5.3.3. Of the 14 peptides tested, peptides with the sequences RATYDK, YEVR, LEDEMK, LMR, DFDGR and SEFAYGSFVR showed antibody binding activity with sensitivity levels between 17 to 36%. To determine whether these peptides

formed linear or conformational antibody epitopes, an inhibition of ELISA was carried out in which each of the peptide was assayed against the other five using the mouse antiserum to 17 kDa antibody. The peptides YEVR and ATYDK were mutually inhibitive thus indicating that they were a part of a complete antibody epitope, which was confirmed also by the determination of the complete structure of the 17 kDa antigen as in section 5.2.2, Fig 4. The other four peptides were linear and probably conformational in the presentation of the antibody epitope. Of the said six antibody epitope bearing peptides, the peptides of the following amino acid sequences were synthesized by solid phase method of Merrifield and were found to contain specific and sensitive antibody binding activities :

RATYDKRYEVR : Sensitivity 65%; Specificity 95%

SEFAYGSFVR : Sensitivity 66%; Specificity 95%

The antibody epitope mapping as described has thus indicated that defined sub structures or peptides of 17 kDa antigen can be synthesized and used for the immunodiagnosis of human tuberculosis in micro ELISA.

5.3.5 Demonstration that 17 kDa antigen is lympho proliferative

Peripheral blood lymphocytes (PBL) from healty donors and TB patients were fractionated and 2 x 10^6 cells were cultured in

presence or absence (control) of 1 ug of 17 kDa antigen for 3 days in RPM1 1640 medium with 10% autologous serum. 24 h before harvesting cultures were pulsed with 1 uCi of 3H thymidine. Table III shows that the 17 kDa antigen was lympho proliferative to the lymphocytes of TB patients (data shown for 2 persons only).

Table III. Lympho proliferative assay with 17 kDa antigen.

Source (PBL)	3H thymidine incorporation (cpm, mean of triplicate cultures)	
Healthy	Control : 90	Antigen : 121
TB patient	Control : 110	Antigen : 650

In addition to the lympho proliferative property of the 17 kDa antigen, a method of prediction of T cell stimulatory epitopes (Rothbard and Taylor, 1988) was used to map the probable sites in the structure of the 17 kDa antigen. These T cell epitopes were located on peptides of the following sequences:

SEFAYGSFVR

AELPGVDPDCDVCITR

Thus this experiment indicates that the 17 kDa antigen or sub structures (peptides) can be used to stimulate human peripheral blood lymphocytes. Since stimulated lymphocytes

elaborate several cellular growth and differentiation factors which contribute to the vaccine effect of 17 kDa antigen or its sub structures, the 17 kDa antigen can be used at the first instance as a vaccine against TB and also for non-specifically boosting cellular immunity.

Figure 6 shows the primary structure of the 17 kDa antigen from M.tuberculosis containing the biologically active regions, although similar activity need not be ruled out in the unmarked regions.

6. Discussion and summary of test results

The present invention describes the immunochemical properties of a novel 17 kDa protein antigen from M.tuberculosis (SII 1 strain). M.tuberculosis causes tuberculosis worldwide among 16 million people. Because of inadequacy of the diagnostic procedures available now the disease has not yet been eradicated. The focus of research in recent years has been the development of immuno diagnostic methods for detecting TB at an early stage as well identification of suitable candidates for vaccination since the traditional BCG vaccine has given only a partial protection against TB.

The studies described in this invention show that a novel 17 kDa antigen derived from M.tuberculosis has antigenic activity. Firstly, it was found to be unique for the South Indian strains of M.tuberculosis. Secondly, its antigenic

nature and chemistry were investigated. Thirdly, the biologically active proteins of the protein antigen were mapped and immuno diagnostic methods were developed for the early detection of TB.

Thus the 17 kDa protein antigen which had 131 amino acids was found to contain two peptides of sequence RATYDKRYEVR and SEFAYGSFVR which carried antibody binding epitopes for diagnosis of TB. Further, it contained two peptides which carried predicted T cell stimulating regions in sequences, SEFAYGSFVR and AELPGVDPDCDVCITR. The latter two peptides presumably contributed to the T cell stimulating property of the whole 17 kDa antigen described in this invention. The T cell stimulating property of the 17 kDa antigen and its sub structure peptides means that they could be used in therapy and vaccination for TB.

7. Figure legends.

Fig. 1. HPLC analysis of the electro eluted 17 kDa protein antigen from M. tuberculosis (SII 1).
HPLC conditions: RP 18 column (LKB, 10 um pore size), A: 0.1% TFA in water, B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% B in 40 min, Sensitivity: 0.08,220 nm.

Fig. 2. Tryptic peptides of 17 kDa protein antigen from M.tuberculosis (SII 1) fractionated by HPLC on RP 18 (LKB,10 um pore size).
Fractionation: A: 0.1% TFA in water, B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% B in 60 min, Sensitivity: 0.08, 220 nm.
Amino acid sequence: Sequence determined are drawn against each peptide.

Fig. 3. V8 protease peptides of 17 kDa protein antigen from M.tuberculosis (SII 1) fractionated by HPLC on RP 18 (LKB, pore size 10 um).
Fractionation: A: 0.1% TFA in water; B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% in 60 min, Sensitivity: 0.082,220 nm.

Fig. 4. The primary structure of the 17 kDa antigen of M.tuberculosis (SII 1) showing the alignment of peptides. Trp: Trypsin, V8: Staphylococcus aureus V8 protease. Superscript arrows denote the amino acid sequence obtained with the whole protein.

(Single letter code used for amino acids).

Fig. 5. The primary structure of the 17kDa antigen from M.tuberculosis (SII 1) showing the biologically active regions.
AA 68 to 77: Antibody and T cell epitopes present.
AA 91 to 101: Antibody epitope present.
AA 107 to 122: Two T cell epitopes present.

8. References

1. A.Hopps and E.J. Woods, Proc.Natl.Acad.Sciences, (1981) 78,3429
2. Hunkapiller, M,W and Lujan E, In, Methods in Protein Microcharacterization, Ed.J.E. Shiveley, Humana Press (1986) pp.89
3. Naganathan, N, etal. Tubercle (1986) 67,261
4. Shinnick,F, et al, J.Bacteriol, (1987) 169,1080
5. Yamaguchi, Y, etal, FEBS Letters, (1987) 240,115
6. Pattorroyo G, et al, Modern Biotechnology in Health, Academic press, (1987), pp.110.
7. Ivanyi, J et al, Springers Seminars Immunopathol, (1988)10 279
8. Abou-Zeid etal, J.Gen Microbiol (1988) 134,531
9. Matsuo M etal, J.Bacteriol, (1988) 170, 3847
10. Rothbard, J,B and Taylor, W, EMBO J (1988) 7,93
11. Ashbridge S, etal, Nucleic Acids Res (1989) 17,1249
12. Boremans et al, Infection and Immunity, (1989) 57, 3123
13. Garcia E, etal (1989) Infection and Immunity (1989),57,204
14. Baird et al, J.Gen Microbiol, (1989)135, 931.

CLAIMS

1. The protein of the structure:

1 40
ATTLPVQRHPRSLFPEFSELFAAFPSFAGLRPTFDTRELM

80
RSIQITIKLEDEMKGIYLPVAKHGELRSEFAYGSFVRTVS

131
LPVGADEDDIRATYDKRYEVRDFDGRAELPGVDPDCDVCITRGILTVSVCV

2. Peptides of the structure:

SEFAYGSFVR

RATYDKRYEVR

AELPGVDPDCDVCITR

and fragments of the protein according to claim 1 which include one or more of these peptide sequences.

3. A DNA sequence coding for the protein defined in claim 1.

4. A DNA sequence coding for peptides defined in claim 2.

5. A DNA or RNA hybridization probe, constructed on the basis of the proteins or peptides defined in claim 1 or 2 or on the basis of DNA sequences according to claim 3 or 4, said probe being optionally labelled.

6. An antibody to a protein or peptide as defined in claim 1 or 2.

7. An antibody according to claim 6 which is a polyclonal or a monoclonal antibody, said antibody being optionally labelled.

8. A kit for performing immuno diagnosis of tuberculosis including a protein or peptide according to claim 1 or 2.

9. A kit for performing immuno diagnosis of tuberculosis including a monoclonal antibody according to claim 7.

10. A kit for performing DNA based diagnosis of tuberculosis including a hybridization probe according to claim 5.

11. A microorganism capable of expressing a protein or peptide according to claim 1 or 2.

12. A vaccine against tuberculosis including a protein or peptide according to claim 1 or 2.

13. An in vitro method of diagnosis of human tuberculosis which comprises interacting body fluids such as serum from a patient with a protein or peptide according to claim 1 or 2.

14. An in vitro method of diagnosis of human tuberculosis which comprises interacting body fluids such as sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody according to claim 7 in labelled form.

15. An in vitro method of diagnosis of human tuberculosis which comprises interacting body fluids such as sputum, CSF, pleural fluid or serum from a patient with a DNA probe according to claim 5 in labelled form.

16. An in vitro method for detection of human tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a polyclonal antibody according to claim 7 in labelled form.

17. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body in immuno-diagnosis, therapy or vaccination in relation to human tuberculosis.

18. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body for producing mammalian polyclonal antibodies or monoclonal antibodies in mice for use in an immunodiagnostic method like sandwich ELISA which detects the protein according to claim 1 among human tuberculous specimens.

19. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body for the detection of antibodies among tuberculous specimens by serological methods for early detection of tuberculosis.

20. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body for the detection of T cell proliferation in a tuberculous specimen for obtaining immuno diagnosis through skin tests or obtaining a candidate for vaccine against

tuberculosis.

21. Use of a protein or peptide according to claim 1 or 2 for developing a reagent for therapy of tuberculous inflammation by T cell proliferation.

22. Use of a protein or peptide according to claim 1 or 2 for the production of growth and differentiation factors using T cell proliferation assay.

23. The use of DNA or RNA probes according to claim 5 for detection of M.tuberculosis DNA among human tuberculous specimen for early detection of tuberculosis.

24. The use of DNA or RNA probes according to claim 5 for the purposes of identification of mycobacterial DNA for example among cultural isolates and for laboratory research.

25. A protein or peptide according to claim 1 or 2 substantially as hereinbefore described.

26. A DNA sequence or a DNA or RNA probe according to any one of claims 3 to 5 substantially as hereinbefore described.

27. An antibody according to claim 6 or 7 substantially as hereinbefore described.

28. A kit according to any one of claims 8 to 10 substantially as hereinbefore described.

29. A microorganism according to claim 11 substantially as hereinbefore described.

30. A vaccine according to claim 12 substantially

as hereinbefore described.

31. A method according to any one of claims 13 to 16 substantially as hereinbefore described.

32. A protein or peptide according to any one of claims 17 to 20 substantially as hereinbefore described.

33. A use according to any one of claims 21 to 24 substantially as hereinbefore described.

Published 1991 at The Patent Office, State House, 66/71 High Holborn, London WC1R 4TP. Further copies may be obtained from Sales Branch, Unit 6, Nine Mile Point, Cwmfelinfach, Cross Keys, Newport, NP1 7HZ. Printed by Multiplex techniques ltd, St Mary Cray, Kent.